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Acute toxicity study of the standardized methanolic extract of *Mitragyna speciosa* Korth in Rodent

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ABSTRACT

Ethnopharmacological relevance: *Mitragyna speciosa* Korth (ketum) is widely used in Malaysia as a medicinal agent for treating diarrhea, worm infestations and also acts as an analgesic and antipyretic.
Aim: The aim of the study is to determine the acute toxicity of *Mitragyna speciosa* Korth standardized methanol extract *in vivo* in 4-weeks-old Sprague–Dawley rats.
Methodology: Rats were orally administrated single dose of 100, 500 and 1000 mg/kg *Mitragyna speciosa* Korth standardized methanol extract and the control group received 430 mg/kg of morphine orally. There were 10 rats in each group. All animals were sacrificed after 14 days of treatment. Eight parameters were tested: cage side observation, body weight measurement, food and water consumption, blood pressure, absolute and relative organ weight, hematology, biochemical analysis and histopathology, to look for evidence of toxicity.
Result: No mortality was noted after 14 days of treatment. In general, behavior, food and water consumption, hematological studies and organ weights showed no significant changes. The standardized methanol extraction of *Mitragyna speciosa* Korth increased rat blood pressure (systolic: 147.4 ± 1.01, 131.64 ± 4.94 and 137.8 ± 4.46) after an hour of 100, 500 and 1000 mg/kg doses, respectively. Biochemical studies showed significant elevation of ALT, AST, albumin, triglycerides, cholesterol and albumin ($p > 0.05$), at all levels of doses. But, nephrotoxicity evidenced by elevated creatinine was seen only at a dose of 1000 mg/kg. Histological examination showed congestion of sinusoids, hemorrhage hepatocytes, fatty change, centrilobular necrosis and increased number of Kuppfer cells in the liver of all *Mitragyna speciosa* Korth standardized methanol extract treated groups.
Conclusion: Oral administration of standardized methanolic extraction of *Mitragyna speciosa* Korth resulted in increasing rat blood pressure after an hour of drug administration. The highest dose of extract also induced acute severe hepatotoxicity and mild nephrotoxicity. However, *Mitragyna speciosa* Korth shows no effects on body weight, food and water consumption, absolute and relative organ weight and also hematology parameters.

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1. Introduction

Mitragyna speciosa Korth or its common name ketum belongs to the family Rubiaceae and sometimes known as Naucleae tribe from subfamily Naucleoideae. Genus mitragyna can usually be found in swamp and valleys in tropical and subtropical Asia such as Thailand, Laos, Cambodia, Malaysia (Burkill, 1936) and in the East and West Africa and in India (Harvala and Hinou, 1988). In Malaysia, this species can be widely found in northern half of Peninsula (Burkill, 1936) and Selangor (Houghton and Said, 1986). *Mitragyna speciosa* is a type of tropical evergreen and non-seasonal plants, which grows heavily in damp areas rich with humus. This species is sensitive towards drought and extreme cold (Shellard, 1974). *Mitragyna speciosa* is one of the medicinal plants used to treat various types of diseases especially in Thailand and Malaysia. According to Kumarnsit et al. (2006), the leaves are the most effective part. It is used as analgesic, antipyretic, antidiarrheal, and local anesthetic; besides it is being used as an energy-boosting agent by farmers (Shellard, 1974; Harvala and Hinou, 1988; Jansen and Prast, 1988). It also can cause other harmful effects including dry mouth, dieuresis, loss of appetite, weight loss, darken skin and constipation (Jansen and Prast, 1988). Despite the widespread studies of *Mitragyna speciosa* done by researches, however, less emphasize has been laid on toxicological effect of this plant. Information about toxicity of this plant is very important as a baseline before exploring further to develop this plant as a new herbal medication. Therefore,

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the purpose of the present study is to determine the acute toxicity effect of *Mitragyna speciosa* Korth standardized methanol extract *in vivo*.

2. Methodology

2.1. Plant material

The *Mitragyna speciosa* leaves were collected from Jengka, Pahang, Malaysia. The plant was harvested and identified by Forest Research Institute Malaysia (FRIM) and conserved at USM herbarium with the voucher number USM 11074. These leaves were thoroughly washed with distilled water to remove dirt. The wet leaves were weighed and then they were dried in an oven at 50 °C for 12 h. During this time, the leaves were periodically turned over to provide uniform drying. The dried leaves were ground to a fine powder by a mill machine and the powder was weighed.

2.1.1. Methanol crude extract preparation

One hundred grams of the powdered leaves was exhaustively soxhlet extracted in absolute methanol (100% v/v MeOH) using an Ace Soxhlet Extractor 6730 and condenser 6740 (Quick fit, England) for 4 h at 60 °C. The brownish extract was then concentrated under reduced pressure at 40 °C using rotary evaporator. The extract was further concentrated by allowing it to stand overnight in an oven at 30 °C to remove trace of methanol solvent. The final product yielded 20.0 g of a green solid methanol extract which was then screened for the presence of the alkaloid mitragynine using GC–MS. From the standard curve, 1.6% of mitragynine was present in the *Mitragyna speciosa* Korth standardized methanolic extract. The dried extract was sealed in a bottle and stored in the refrigerator at 4 °C until further use.

2.1.2. Standardize extraction

The *Mitragyna speciosa* leave extraction was standardized with reference to the amount of mitragynine content using validated GCMS method. Dried extract was stored at 4 °C and its stability was monitored for 12 months.

2.2. Animals

Adult male albino rats (Sprague–Dawley) weighing 150–200 g were obtained from the breeding colony of the Laboratory Animal Research Unit (LARU), Universiti Sains Malaysia (USM). They were housed one rat per cage and maintained at a constant temperature on a standard 12:12 light/dark cycle with light on at 7 a.m. Food and water were available *ad libitum* in the normal acrylic cage. The experiments were conducted according to the ethical norms approved by Universiti Sains Malaysia, Kubang Kerian Health Campus Animal Ethics Committee guideline for animal care.

2.3. Extract and drug administration

The dried methanol extract was insoluble in saline solution; hence it was re-suspended using 1% carboxyl-methyl-cellulose (CMC) as a vehicle. The suspension was then stored at 4 °C until further use (Amresh et al., 2007). The standardized methanol crude extract of *Mitragyna speciosa* with various concentrations (100, 500 and 1000 mg/kg body weight) and morphine with toxic concentrations (430 mg/kg body weight) were homogenously mixed together with CMC and administrated orally using gavage needle to five groups of animals.

2.4. Dosage of extract on animals

Single dose toxicity studies were carried out by adapting OECD guideline nos. 420 (OECD, 2001) and 407 (OECD, 1995). In order to study any possible toxic effect or changes in normal behavior, 10 groups of rat (all males) were used in this experiment. The acute toxicity of the ethanol extract was studied by preparing three different concentrations of the standardized extract (as above) and administrated orally to three groups of animals. Morphine with toxic concentrations was used as a positive control group. The fifth group was taken as a negative control and given as vehicle (1% CMC in distilled water). The maximum volume of CMC administered was not greater than 2 mL/100 g body weight. Animals were fasted prior to conducting the experiment (only food but not water was withheld overnight). Following the 24 h of fasting, the animals were weighed and the test substance was administered. After the substance has been administered, food may be withheld for a further 3–4 h.

2.5. Cage side observation and body weight measurement

In this study, animals were observed individually after drug administration and special attention was given during the first 4 h and every 12 h daily thereafter, for a total of 14 days. All observations were systematically recorded, with individual records being maintained for each animal. Cage side observation included evaluation of skin and fur, eyes, respiratory effects, autonomic effects such as salivation, diarrhea, urination, and the central nerve effects including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli and altered strength (Kumarnsit et al., 2006; Demma et al., 2006; Mukinda and Syce, 2007; Obici et al., 2008). Individual body weight was measured and recorded prior to the administration of extracts and drug for 2 weeks. Weight changes were also calculated and recorded.

2.6. Food and water consumption

The amount of food and water consumed was measured daily from the quantity of food and water supplied and the amount remaining after 24 h for 2 weeks of the study period (Kumarnsit et al., 2006; Demma et al., 2006; Obici et al., 2008).

2.7. Blood pressure

Blood pressure (systolic and diastolic) of rats in each group was measured by the noninvasive tail cuff method. The animals were trained for blood pressure measurement for three consecutive days. Each conscious rat restrained in a warmed 28 °C incubator before drug administration. This was done to familiarize the rats to the system and also to ensure the rats are calm and comfortable during the actual measurement. Furthermore, this procedure may eliminate bias and data inaccuracy. The actual measurement was conducted an hour after drug administration (Mahinda et al., 2004).

2.8. Absolute and relative organ weight

At the end of the acute toxicity study (14 days), all organs were collected using standardized surgical procedures. The abdominal cavity of each animal was dissected and organs namely the heart, liver, lungs, spleen, kidney and brain were quickly removed, cleaned with saline, weighed and preserved in 10% formalin for histopathology analyses (Demma et al., 2006; Obici et al., 2008; Tan et al., 2008).

Table 1
Blood pressure recordings of rats following single dose administration of negative control, morphine and *Mitragyna speciosa* Korth extract groups. Data indicate mean ± SEM.

Parameter	Negative control (CMC) $n = 10$	Positive control (morphine) $n = 8$	MS 100 mg/kg $n = 10$	MS 500 mg/kg $n = 10$	MS 1000 mg/kg $n = 10$
Systolic	120.6 ± 0.91	109 ± 4.25[a]	147.4 ± 1.01[a,b]	131.64 ± 4.94[a,b]	137.8 ± 4.46[a,b]
Diastolic	80 ± 1.15	67.5 ± 2.72[a]	102.7 ± 0.72[a,b]	98.74 ± 7.95[a,b]	86.85 ± 3.34[a,b]

Abbreviation: MS, *Mitragyna speciosa* extract.

[a] Statistically different from negative control (CMC), $p < 0.05$.

[b] Statistically different from positive control (morphine), $p < 0.05$.

2.9. Hematology

At the end of the acute toxicity study (14 days), all the animals were anesthetized using chloroform and bled via inferior vena cava puncture. The samples were collected in plastic test tubes containing EDTA anti-clotting. Hematology analyses were performed on whole blood, using the automatic hematology system, ADVIA® 60 open tube (Bayer Corporation, USA) to evaluate the following parameters: erythrocyte count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count and leucocyte count (WBC) (Demma et al., 2006; Obici et al., 2008; Tan et al., 2008).

2.10. Biochemical analysis

At the end of the acute toxicity study (14 days), all the animals were anesthetized using chloroform and bled via inferior vena cava puncture. The sample were collected in plastic test tubes and allowed to stand for complete clotting. The clotted blood samples were centrifuged at 3000 rpm for 15 min and serum samples were aspirated off and frozen at −80 °C. Serum sample were analyzed for the determination of total protein, urea, creatinine(CREA), glucose, total cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphate (ALP), albumin (ALB), bilirubin (BIL), Lactate dehydrogenanse (LDH), and gamma-glutamyltranspetidase (GGT) (Demma et al., 2006; Mukinda and Syce, 2007; Obici et al., 2008; Tan et al., 2008).

2.11. Histopathological observation

The four organs of all animals (liver, kidney, lung and brain) were examined macroscopically. The morphology of the internal organs was visually observed for signs of toxicity. These organs have undergone hematoxylin and eosin (H&E) staining. Abnormalities of the slides were graded as mild, moderate and severe (Jain et al., 2008; Tan et al., 2008).

2.12. Statistical analysis

Non-parametric one-way statistical analysis of variance, Kruskal–Wallis test and post-hoc test (Mann–Whitney test) were used in order to see any significant changes between *Mitragyna speciosa* extract groups, morphine group (positive control) and CMC group (negative control). Differences between groups were considered significant at $p < 0.05$. All data points show the mean of standard error of means (SEM).

3. Result

3.1. Acute toxicity study

3.1.1. Cage side observation

Oral administration of *Mitragyna speciosa* extract in doses of 100 and 500 mg/kg did not produce significant changes in behavior, skin effect, breathing, defecation, postural abnormalities, impairment in food intake and water consumption and yellowing or loss of hair. Rats treated with 1000 mg/kg *Mitragyna speciosa* extract showed very low level of activity (slow movement) and rapid breathing which lasted 30 min after drug administration. No mortality of animal was observed in *Mitragyna speciosa* extract treated groups. However, based on the physical and physiological observations, animals under positive control group treated orally with toxic level of morphine showed changes when compared to other groups (negative control and treated groups). Rats treated with morphine were less responsive to external stimuli, motor activities were reduced, breathing was slow and eyes were red, lasting up to 30–45 min with drug administration. Two rats died in less than 24 h of post-treatment.

3.1.2. Body weight measurement

The mean of rats' body weight was measured daily for 14 consecutive days. CMC (negative control) group showed the highest increase in body weight compared to the other four rat groups administered with morphine and *Mitragyna speciosa* extract. Rats administered with morphine showed the least change in body weight.

3.1.3. Food and water consumption

The amount of food and water consumed was measured daily from the quantity of food and water supplied and the amount remaining after 24 h. Values for consumption are based on total intake and average relative body weight of the preceding time interval. Each data represent the value of $n = 10$ for each group except for morphine, $n = 8$. These data showed no statistically significant difference from negative control (CMC), $p > 0.05$.

3.1.4. Blood pressure

Blood pressure (systolic and diastolic) recording in animal samples is shown in Table 1. Based on the results, rats from morphine group showed significantly low reading compared to negative control group. All groups of *Mitragyna speciosa* extract exhibit significantly high blood pressure for both systolic and diastolic compared to negative control group and positive control group (morphine).

3.1.5. Absolute and relative organ weight

Absolute and relative organ weights of rats were measured at the end of acute toxicology test period (14 days). The values for each organ represent the value of $n = 10$ for each group except for morphine, $n = 8$. No statistically significant differences (Kruskal–Wallis analysis) were shown among groups, $p > 0.05$.

3.1.6. Full blood count (FBC) test

Hematological data of rats for seven parameters were analyzed at the end of acute toxicology test period (14 days). The values for full blood count (FBC) test for each data represent the value of $n = 10$ for each group except for morphine, $n = 6$ and 100 mg/kg MS, $n = 8$. No statistically significant difference was shown among groups.

Table 2
Biochemistry data of rats after single dosage of negative control, morphine and *Mitragyna speciosa* Korth standardized methanol extract. Data indicate mean ± SEM.

Parameter	Negative control (CMC) $n = 10$	Positive control (Morphine) $n = 8$	MS 100 mg/kg $n = 10$	MS 500 mg/kg $n = 10$	MS 1000 mg/kg $n = 10$
Urea (mmol/L)	4.45 ± 0.52	7.31 ± 0.34[a]	7.07 ± 0.29[a]	7.50 ± 0.44[a]	6.64 ± 0.20[a]
Creatinine	23.56 ± 4.40	48.895 ± 2.37[a]	43.08 ± 1.58[a]	50.88 ± 2.55[a]	44.10 ± 0.93[a]
GGT (U/L)	16.02 ± 1.03	18.08 ± 0.34	16.73 ± 0.36	18.00 ± 0.55	16.64 ± 0.50
AST (U/L)	68.59 ± 10.69	142.04 ± 4.73[a]	132.06 ± 7.39[a]	124.53 ± 11.24[a]	125.17 ± 3.60[a]
Albumin (Abs)	20.41 ± 2.40	38.90 ± 0.73[a]	39.43 ± 0.37[a]	40.33 ± 0.43[a]	39.33 ± 0.38[a]
ALT (U/L)	39.93 ± 7.10	67.33 ± 3.77[a]	68.58 ± 2.46[a]	67.06 ± 3.69[a]	73.10 ± 2.79[a]
Total bilirubin (μmol/L)	1.20 ± 0.18	1.05 ± 0.21	1.02 ± 0.14	1.18 ± 0.16	1.25 ± 0.11
LDH (U/L)	883.03 ± 108.62	1429.72 ± 88.50	1386.83 ± 164.92	1182.94 ± 147.66	1093.25 ± 97.44
Triglyscerides (mmol/L)	0.26 ± 0.07	1.17 ± 0.20[a]	0.70 ± 0.02[a]	0.72 ± 0.08[a]	0.84 ± 0.08[a]
Cholesterol (mmol/L)	0.49 ± 0.11	1.44 ± 0.02[a]	1.35 ± 0.03[a]	1.41 ± 0.08[a]	1.22 ± 0.04[a]

Abbreviation: MS, *Mitragyna speciosa* extract.

[a] Statistically significant difference compared to the negative control (CMC), $p < 0.05$.

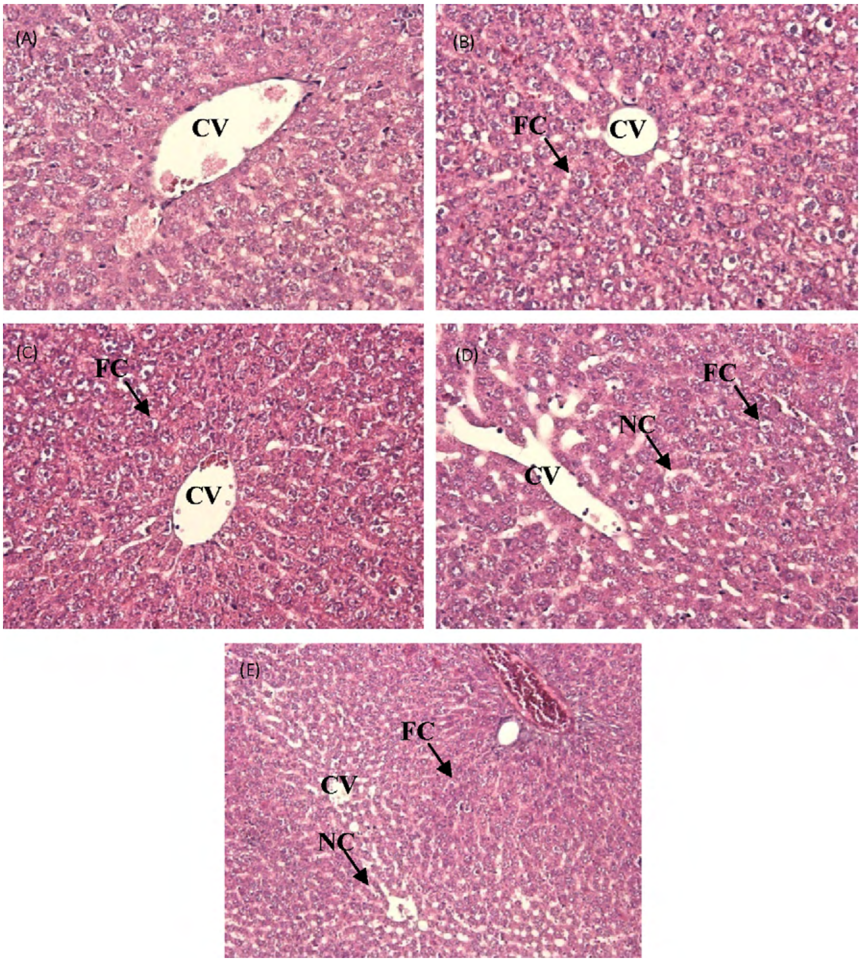

Fig. 1. Liver sections were stained with hematoxylin and eosin (H&E-stained 400×) showing the effects of *Mitragyna speciosa* Korth extract on acute toxicity study of rodent. (A) Negative control (CMC); (B) MS 100 mg/kg; (C) MS 500 mg/kg; (D) MS 1000 mg/kg; (E) positive control (morphine). *Abbreviation*: MS, *Mitragyna speciosa* extract; CV, central vein; FC, fatty change; NC, centrilobular necrosis.

3.1.7. *Biochemical analysis*

The data in Table 2 showed an elevation of readings for the various biochemical parameters for most of the rat groups. Significant reductions were recorded for Urea, CREA, AST, ALB, ALT, triglycerides and cholesterol. The values for biochemistry analysis for each data represent the value of $n = 10$ for each group except for morphine, $n = 8$.

3.1.8. *Histopathology*

Histopathology results showed that *Mitragyna speciosa* extract at 1000 mg/kg as well as morphine caused a significant damage in liver as revealed by severe congestion of sinusoids, hemorrhage hepatocytes, fatty change (lipid accumulation), centrilobular necrosis and numerous Kuppfer cells (Fig. 1). However, there are no significant morphological changes detected in kidney, lung and brain (cortex of frontal lobe and hippocampus) in all animal samples from all groups of study.

4. Discussion

Herbal medicine, a natural remedy has become universally popular in primary healthcare, particularly in developing countries including Malaysia. Herbal sources from medicinal plants are presumed to be safe without any compromising health effect, thus widely used as self-medication (Obici et al., 2008). However, since there is a lack of proven scientific studies on the toxicity and adverse effect of these remedies, further investigations are vitally needed and the same applies for *Mitragyna speciosa*.

In this 14-day period of acute toxicity evaluation, rats given *Mitragyna speciosa* extract at dose 100, 500 and 1000 mg/kg, showed no mortality and all rats did not produce any symptom of toxicity. In the previous study, Jansen and Prast (1988) found that mitragynine (*Mitragyna speciosa* major biocompound) showed no evidence of tremors and convulsions in mice given dose as high as 920 mg/kg body weight. Our result on cage side functional observations such as respiratory effect, autonomic and nervous system effect in high dose group animals showed slight changes in animal behavior but are quickly reversible. The reversibility of a toxic effect is an indicator of how the organism metabolizes the drug (Eaton and Klaassen, 1996). Findings discovered by Reanmongkol et al. (2007) showed that methanol and alkaloid extract significantly prolonged the latency of nociceptive response on tail flick test in rats and suggested that the extracts of *Mitragyna speciosa* leaves possess the analgesic activity which partly acted at opioid receptors in the supraspinal opioid system. However both of the extracts showed no significant changes on spontaneous motor activity or pentobarbital-induced sleep in mice.

Regardless of the dose used, the *Mitragyna speciosa* extract did not appear to affect the body weight of the rats and caused no significant changes in their food and water consumption. Utilization of food and water exhibited normal metabolism in the animals (Mukinda and Syce, 2007) and this suggests that the single oral dose of *Mitragyna speciosa* extract did not retard the growth of rats. The increase of body weight is may be due to the process of body fat accumulation. Rats treated with morphine showed decreased body weight increment. Opioid receptor agonist namely morphine leads to decreased food consumption since it induces the feeling of fullness to the ingestion system (Tsuchiya et al., 2002). In addition, morphine also caused weight loss associated with inhibition of neurons in the lateral hypothalamus (Tsuchiya et al., 2002).

Our finding indicates that all animals from *Mitragyna speciosa* extract groups showed a significant increase in blood pressure compared to both negative and positive control groups after 1 h measurement (single measurement). Since there are a few publications available on the effects of *Mitragyna speciosa* extract on cardiovascular, further investigation should be conducted. Series of measurements with 30 min time intervals for a longer period of time would be appropriate to determine the effect is either transient or long-lasting. In contrast, our result with morphine revealed significant hypotensive effect compared to negative control group. This result is in agreement with previous findings (Gomes et al., 1976; Mahinda et al., 2004; Bodnar and Klein, 2006; Bodnar, 2007).

There were also no significant changes in the absolute organ weights of the heart, liver, kidneys, brain, lung and spleen of the rats treated with *Mitragyna speciosa* extract compared to negative control group. But when organ weights were expressed relatively to body weight, liver of rat treated with morphine showed significant increase. The detectable relative weight changes in liver may reveal the toxicity of morphine to the liver. Relative weight is more indicative of toxicity than absolute weight (Demma et al., 2006), which is further supported by biochemical findings and noticeable effect on gross and histological evaluation conducted in liver groups treated with morphine (refer to Fig. 1).

Biochemistry results showed significant elevation in ALT levels at toxic dose of morphine and at all doses of *Mitragyna speciosa* extract. Elevation of ALT in the morphine group is similar to the observation by Atici et al. (2005). The significant changes in levels of albumin and triglycerides in this study indicate impairments to the hepatic function and liver cell injury (Graca et al., 2007). From this study, the increased level of LDH in the group receiving morphine compared to control groups is in accordance with the findings by Atici et al. (2005).

In this study after 14 days of acute toxicity evaluation of *Mitragyna speciosa* extract to rats, there were no changes to the WBC, RBC, HGB, HCT, MCV, and MCHC. This study revealed that *Mitragyna speciosa* extract is not toxic to the blood parameters.

Histological examination of the *Mitragyna speciosa* extract treated samples of liver stained with hematoxylene and eosin (H&E) revealed some abnormal morphology characteristics in all *Mitragyna speciosa* extract groups. Almost all of the samples treated with *Mitragyna speciosa* extract have revealed the presence of Kupffer cells and enlargement of the nucleus or karyomegaly (Fig. 1). Morphological changes are shown in areas of migrated nuclear chromatin in some of the treated samples. However *Mitragyna speciosa* extract at dose 100 and 500 mg/kg was less severe compared to the highest dose of *Mitragyna speciosa* extract (1000 mg/kg) and morphine. Most of these characteristics observed were also shown in studies of other opiates including morphine (Klaassen, 2001). The effect of morphine on liver exhibits numerous numbers of sinusoids, which comprise Kupffer cells. Impairment to the Kupffer cells by morphine may disrupt its ability in degrading and ingesting particulate matter (Klaassen, 2001). A study conducted in the rats treated with morphine revealed necrosis, hemorrhage, sinusoidal dilatation, apoptotic body, congestion and hydrophic degeneration. Nogamatsu et al. (1986) demonstrated that the addiction of morphine induced a marked decrease in the rat hepatocytes and resulted in cell death. The same effects also are seen in the samples from group of the highest dose of *Mitragyna speciosa* extract.

Although biochemical studies revealed significant increase in cholesterol, urea and creatinine in all *Mitragyna speciosa* extract and morphine groups, histological findings did not support the occurrence of cell damage in kidney. This may be due to the earlier phase of kidney damage (Lin et al., 2007). Increased level of LDH was associated with lung lesion in rats, however, our study found that LDH level is not significant statistically in all groups of animals and from histological findings.

Based on the brain's histopathological results, this study observed no cell or neuron loss in rats from all groups of study. Furthermore, no regional progression to the lesion was observed from the cortex and the hippocampus. Moreover, from the H&E stained brain sections, the hippocampus showed no injury to the white matter. This finding indicated that *Mitragyna speciosa* Korth acute treatments did not induce damage in axons and dendrites of the hippocampal neurons.

5. Conclusion

In conclusion, the standardized methanolic extraction of *Mitragyna speciosa* Korth increased rat blood pressure after an hour of drug administration. The highest dose of extract also induces acute severe hepatotoxicity in rat liver and mild nephrotoxicity in kidney after single dose administration. This was evidenced mainly by histopathological study on all particular organs and has been supported by biochemistry findings. However, *Mitragyna speciosa* Korth shows no effects on body weight, food and water consumption, absolute and relative organ weight and also hematology parameters. This study provides valuable data on the toxicity profile of *Mitragyna speciosa* Korth that should be essential for future study. Further studies may focus on chronic toxicity studies of *Mitragyna speciosa* Korth in order to evaluate its long term effects.

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